

Structural studies of a phosphocholine substituted β -(1,3);(1,6) macrocyclic glucan from *Bradyrhizobium japonicum* USDA 110

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In our previous *in vivo* ³¹P study of intact nitrogen-fixing nodules (Rolin, D.B., Boswell, R.T., Sloger, C., Tu, S.I. and Pfeffer, P.E., 1989 Plant Physiol. 89, 1238–1246), we observed an unknown phosphodiester. The compound was also observed in the spectra of isolated bacteroids as well as extracts of the colonizing *Bradyrhizobium japonicum* USDA 110. In order to characterize the phosphodiester in the present study, we took advantage of the relatively hydrophobic nature of the material and purified it by elution from a C-18 silica reverse-phase chromatography column followed by final separation on an aminopropyl silica HPLC column. Structural characterization of this compound with a molecular weight of 2271 (FAB mass spectrometry), using ¹³C-¹H and ³¹P-¹H heteronuclear 2D COSY and double quantum 2D phase sensitive homonuclear ¹H COSY NMR spectra, demonstrated that the molecule contained β -(1,3); β -(1,6); β -(1,3,6) and β -linked non-reducing terminal glucose units in the ratio of 5:6:1:1, respectively, as well as one C-6 substituted phosphocholine (PC) moiety associated with one group of (1,3) β -glucose residues. Carbohydrate degradation analysis indicated that this material was a macrocyclic glucan, (absence of a reducing end group) with two separated units containing three consecutively linked β -(1,3) glucose residues and 6 β -(1,6) glucose residues. The sequences of β -(1,3)-linked glucose units contained a single non-reducing, terminal, unsubstituted glucose linked at the C-6 position and a PC group attached primarily to an unsubstituted C-6 position of a β -(1,3)-linked glucose.

Introduction

Bacteria of the genus *Rhizobium* are well known for their ability to infect higher plants. This infection process leads to a beneficial symbiotic relationship in which developed root nodules of leguminous plants participate in atmospheric nitrogen fixation. Related to their infective ability there is a growing interest in the carbohydrate molecules produced by these microorganisms. These cell surface carbohydrates include extracellular polysaccharides, capsular polysaccharides, lipopolysaccharides and periplasmic glucans. In *Rhizobium* (fast growing species), the glucan fraction generally is

composed of neutral cyclic β -(1,2) glucan oligosaccharides [1–3]. Often these periplasmic oligosaccharides produced by the *Rhizobium* sp. are similar to those produced by *Agrobacterium* [4,5] and can contain from 17 to 33 glucose units [6]. Some of these cyclic β -(1,2) glucans are substituted with anionic phosphoglycerol in one strain [7,8] and methylmalonate or succinic acid in others strains [9]. Recently, Miller et al. [10] have provided evidence for a role of cyclic β -(1,2) glucans in osmoregulation of gram-negative bacteria. In addition, Tully et al. [11] have shown the same effect with β -(1,3);(1,6) glucans. The synthesis of these saccharides was reduced when cells were grown under high osmotic potential. On the other hand, Nester et al. [12,13] and Geremia et al. [14] provided evidence that cyclic β -(1,2) glucans play a part in the plant infection process. In these studies, *Rhizobium meliloti* was ineffective in colonizing alfalfa and failed to produce cyclic β -(1,2) glucan. After addition of cyclic β -(1,2) glucan (isolated from the periplasm of *Rhizobium trifolii*) clover root infection thread formation was increased by 96% [15].

Early work of Dudman and Jones [16] demonstrated that the *Bradyrhizobium japonicum* strain 3I1b71a (slow growing species) produced extracellular mixed β -(1,3) and β -(1,6) glucans in two molecular weight ranges (4500 and 12000). More recently, extracts of *B. japonicum* USDA 100, USDA 94 and *Bradyrhizobium* sp. strain 32H1 reported by Miller et al. [17] revealed the presence of an unsubstituted β -(1,3);(1,6) cyclic glucan containing 11–13 glucose units per ring. In this report, we describe the detailed structural features of a phosphocholine (PC) substituted β -(1,3);(1,6) macrocyclic glucan found in *B. japonicum* USDA 110 as well as intact nitrogen-fixing nodules and speculate on its possible biological function.

Materials and Methods

Bacterial strains and culture conditions

B. japonicum USDA 110, USDA 138, USDA 24, *R. leguminosarum* biovar *viceae* (strain 2370), biovar *trifolii* (strain 2152), biovar *phaseolii* (strain 2676), *R. meliloti* 1020a and *Bradyrhizobium* sp. *lotus* (strain 3074) were provided by C. Sloger of the Cell Culture and Nitrogen Fixation Laboratory, Agricultural Research Service, Beltsville, MD. 12-l cultures of each strain were grown in medium containing 2 g of arabinose, 1.3 g of Hepes, 1.1 g of Mes, 1 g of yeast extract, 0.32 g of NH_4Cl , 0.25 g of Na_2SO_4 , 0.18 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.125 g of Na_2HPO_4 , $1.5 \cdot 10^{-2}$ g of CaCl_2 and $0.67 \cdot 10^{-2}$ g of FeCl_3 per l (pH 6.8) at 30°C on a rotary shaker. Soybean (*Glycine max* (L.) Merr, cv Williams) were inoculated with *B. japonicum* USDA 110 and raised in a growth chamber as described previously [18]. Nodules were harvested 35–55 days after inoculation.

Extraction of cell-associated oligosaccharides

Bacteria were harvested during the logarithmic growth period by centrifugation ($16000 \times g$), washed with distilled water once, and quickly frozen in liquid nitrogen. The cells were ground in a mortar in the presence of perchloric acid (0.8 M final concentration) and liquid nitrogen. The mixture was warmed up to 0°C and then frozen again with liquid nitrogen (three cycles). Sonication was performed at 4°C with a Sonicator Cell Disruptor (Heat Systems Ultrasonic) for 2 min at output control 7 with 50% pulsed cycle. The thick homogenate, pH adjusted to 6.9, was centrifuged at $12000 \times g$ for 10 min to remove KClO_4 . The resulting supernatant was lyophilized, redissolved in distilled water and dialyzed for 4 days against distilled water with dialysis tubing (molecular weight cutoff 1500 Spectrapor *, Spectrum Medical Industries). 12 g of

soybean nodules were extracted in the same manner to obtain the polysaccharide mixture for further analysis.

Purification of cell-associated oligosaccharides

Purification of the material was effected, due to its 'hydrophobic' nature by elution on a C18 silica reverse-phase chromatography column (Worldwide Monitoring, Horsham, PA). Before application of each sample (100 mg) to the C18 silica (20 g of C18 silica/g of dry extract), the column was washed with methanol (50 ml) followed by water (500 ml). After elution of the sample through the C18 silica at a concentration of 2 g/l with water, the column was washed with 200 ml of water. The material was eluted off the C18 silica with 40 ml of a 30% methanol solution. The progress of the elution was monitored by ^{31}P -NMR spectroscopy. Following freeze-drying the product was dried to constant weight at 60°C in a vacuum oven for 15 h.

HPLC analysis of the saccharide mixture

2–4 mg of the sample obtained from the C-18 column elution with 30% methanol was dissolved in water and chromatographed on a Hewlett Packard model 1090 HPLC fitted with a 5 μm LC-NH2 (Supelco, Belmont, PA) column (25 cm \times 4.6 mm) and Hewlett Packard 1037 refractive index detector. The column was eluted with water/acetonitrile (43/57) at a flow rate of 1 ml min $^{-1}$ at ambient temperature. Typically, 30 μl of sample (60–120 μg) was injected.

Oligosaccharides analysis by gel-filtration

After purification, the freeze-dried material was dissolved in water (2 ml) and applied to a column of Sephadex G-100 (2.5 by 97 cm) which was standardized with 3 standards 74 K dextran, 15–20 K dextran and glucose. The column was eluted at room temperature at a rate of 22 ml/h with water. Fractions (4.7 ml) were collected and assayed for total carbohydrates by the Phe- H_2SO_4 method [19]. The molecular weight of the glucan was also estimated by gel-filtration chromatography on a Bio-Gel P-4 (Bio-Rad, Richmond, CA) column (1 \times 100 cm). An aliquot of the freeze-dried material was applied and, the column was eluted at room temperature with 0.15 M ammonium acetate (pH 7.0) in 7% (v/v) propanol. Fractions of 1.5 ml were collected at a flow rate of 3 ml/h. The material was detected using the phenol- H_2SO_4 method [19]. The column was calibrated with the following molecular weight standards: glucose, sophorose, cyclohexaamylose, cyclooctaamylose and cyclic β -(1,2) glucan standard (mol. wt. = 2200, $n = 14$ and 3100, $n = 19$, prepared from *Agrobacterium tumefaciens* C58 as described by Miller et al. [10]). The carbohydrate standards were detected using the Phe- H_2SO_4 method [19]. K_{av} was calculated as $V_e - V_0 / V_t - V_0$ where V_e is the elution volume, V_0 is the void volume and V_t is

the total volume. The mol. wt. of the unknown was estimated by constructing a standard curve of K_{av} vs. log of the molecular weight.

In vivo NMR experiments

Detached nodules (2–3 g) were split in half with a razor blade and transferred to a 10 mm NMR tube equipped with a perfusion system similar to that described earlier by Rolin et al. [18]. The 161.7 MHz ^{31}P -NMR spectra (obtained with a 54 mm narrow bore magnet JEOL CX 400 MHz) were accumulated at $22^\circ\text{C} \pm 1$ over a 16000 Hz frequency range with 2000 data points zero filled to 16000, 20 k transients and a repetition time of 0.162 s (total accumulation time of 54 min) utilizing approx. a 30° pulse (12 μs) with low power complete proton decoupling. The bacteroid isolation was basically the same as described by Tajima et al. [20]. The bacteroids were examined in dialysis tubing within the NMR tube. Glass wool was used to maintain the bacteroids in suspension during the experiment. A reference capillary containing 120 mM HMPA* was used to give a satisfactory size reference peak. The HMPA exhibited a resonance of 30.73 ppm downfield from 85% H_3PO_4 .

1D- and 2D-NMR of the cell associated oligosaccharides

High resolution 1D ^{31}P -NMR, inverse detected phase-sensitive ^1H - ^{13}C correlated 2D, and double quantum filtered phase-sensitive ^1H homonuclear COSY 2D spectra of the sample were recorded at ambient temperature on a Bruker AM-500 spectrometer operating at 202.4, 125.8 and 500.1 MHz for ^{31}P , ^{13}C and ^1H , respectively. The 2D ^{31}P - ^1H COSY spectrum of the sample was recorded on a Bruker AM-400 MHz spectrometer with a 10 mm broadband probe and a 1.1 ml insert. The freeze-dried sample was dissolved in H_2O or in D_2O as indicated at a concentration of approx. 25 mg/ml.

The ratio of $(\text{CH}_3)_3\text{N}^+$ /anomeric carbon (glucose units) and the β -(1,3) to (1,6) glucose ratio was obtained from the 100.4 MHz ^{13}C -spectrum of the glucan (25 mg/ml) under quantitative conditions, i.e., inverse gated decoupling, spectral width of 25 kHz, 9820 scans, 16 K data points, a 90° pulse angle and a repetition rate of 5 s. The choline content was determined by comparing the area of the choline resonance at 55.19 ppm (normalized for one group) with the anomeric resonances centered at approximately 104 ppm. To obtain the β -(1,3);(1,6) glucose ratio we compared the area of the free CH_2OH resonance at 61.94 ppm with that of the anomeric resonances as well as the C_3 (1-3) resonances centered at 85.60 ppm.

^{13}C -spectra of the Smith degradation products were examined at 100.4 MHz in a 0.75 ml microcell using a 10 mm broadband probe. The 3 mg sample required 40 K scans, 80° pulse angle and 1.38 s repetition rate to obtain a spectrum with sufficient S/N to determine the degree of choline substitution.

Analysis of the phosphorus content of each weighed sample was carried out in a 1.1 ml microcell. The 161.7 MHz ^{31}P -spectra were obtained under quantitative conditions, 90° pulse, repetition time of 6 s ($T_1 = 1.0$ s) and 16 scans. The spectral width was 4 kHz with 8 K data points zero filled to 16 K. These spectra were normalized to the spectrum of 5 mM P_i taken in the same volume microcell under the same conditions as above, except that a longer repetition time (41 s) was required due to the longer T_1 (6.8 s) of P_i . A solution containing 19 mg of compound corresponded to a value of 6.3 mM phosphorus in the 1.1 ml microcell volume gave an approx. mol. wt. of 2500.

Glycosidic linkage analysis

Samples were permethylated, subjected to acid hydrolysis, derivatized as the alditol acetates and subsequently analyzed by gel-filtration chromatography (GLC)-mass spectrometry [21].

Smith degradation

20 mg of compound was added to a 5 ml solution of 50 mM NaIO_4 and stored at 4°C in the dark for 90 h; 150 mg of ethylene glycol was added and after 30 min, 25 mg of NaBH_4 [22]. The solution was allowed to stand at room temperature for 6 h, then neutralized with 2 M HOAc . The sample was concentrated under N_2 , taken up in MeOH and blown down under N_2 three times to remove borate. The sample was taken up in 1 ml H_2O and fractionated by GLC on a G-15 Sephadex column (1×27 cm) with 20 mM NH_4OAc (pH 6.3). Fractions (2.8 ml) were collected and carbohydrate eluted in fractions 7–10 (14–28 ml) were combined and lyophilized. ^{13}C -NMR was obtained with a 0.75 ml microcell in a 10 mm probe at 100.4 MHz (see NMR Experimentation).

The oxidized product was treated with 1 M TFA at room temperature for 40 h, concentrated to dryness and fractionated on G-15 Sephadex column (200×1 cm) with 1.8 ml fractions with the product eluting in fractions 16–23 (approximate elution of maltotriose). HPLC of a 0.5 M H_2SO_4 hydrolysate of combined fractions 16–23 on a Ca^{2+} Bio-Rad column, 0.3 ml $\text{H}_2\text{O}/\text{min}$ gave a ratio of glucose/glycerol of 2.85/1.

End group analysis

1 mg of compound was reduced with NaBH_4 , hydrolyzed and the aldonitrile derivative prepared in the usual manner [21]. GLC showed no alditol acetate only

aldononitrile acetate of glucose indicating that no reducing glucose unit was present in the sample.

Determination of choline by the choline oxidase method

The presence of choline was determined by an enzymatic method after hydrolysis of 17 mg of phosphocholine substituted β -(1,3);(1,6) cyclic glucan in boiling 0.5 M NaOH for 1 h 20 min. The enzyme incubation mixture contained 20 μ l of 4-aminoantipyrine (60 mM), 20 μ l of phenol (200 mM) and about 5.5 units of horseradish peroxidase (EC 1.11.1.7) (Sigma Type VI Sigma, St. Louis, MO) together with substrates to be tested in 0.05 M Tris buffer of pH 8.0 in a final volume of 2.1 ml. The optical absorbance at 500 nm was read and recorded. The reaction was then started by the addition of 2.7 units of choline oxidase purified (EC 1.1.3.17) (Sigma) dissolved in 0.05 M Tris buffer (pH 8.0). After 45 min at 24°C, an increase of absorbance at 500 nm was read and compared with that of appropriate controls and standards.

Mass spectral analysis

Mass spectra were obtained with a VGZAB-2SE/FPD magnetic sector mass spectrometer (VG Analytical, Manchester, UK) by fast atom bombardment ionization at 8 keV using a thioglycerol/MNBA matrix. An exponential downscan from 4000 to 100 a.m.u. at a scan rate of 15 s/decade with a 2 s reset time was used.

Results

In our previous study [18] we investigated the methodology for examining the symbiotic state between soybean and *B. japonicum* USDA 110 using in vivo ^{31}P -NMR spectroscopy. Different experimental conditions were used to optimize the spectrum and maintain perfused, respiring, detached nodules in the NMR spectrometer magnet. A representative 161.7 MHz in vivo ^{31}P -spectrum of detached, split soybean root nodules is presented in Fig. 1. Peaks in the ^{31}P -NMR spectrum were identified from a proton decoupled ^{31}P -NMR spectrum of a perchloric acid extract of soybean root nodules (not shown). Samples prepared in this manner had well resolved resonances which facilitated the identification of each component. The following peaks were easily identified: G-6-P, F-1,6-diP, choline-P, P_i , NTP, NDP, UDPG and NAD^+ . An unidentified peak (X) in the phosphodiester region (0.44 ppm) was also observed. Its resonance did not correspond to any of the expected phosphodiesters such as glycerophosphocholine, G-P-inositol, G-P-serine, G-P-ethanolamine or G-P-glycerol or phosphomonoester as phosphoenolpyruvate that have resonances in this area of the spectrum. The unidentified compound has been found to be exclusively associated

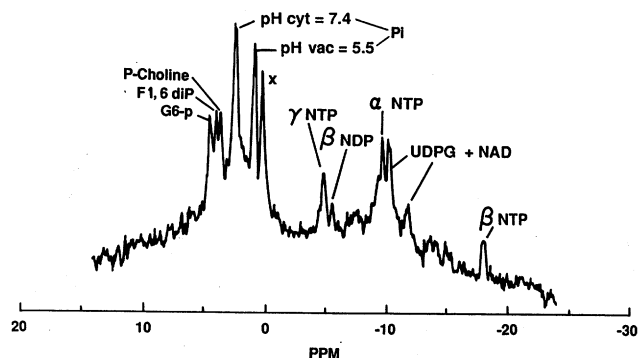


Fig. 1. 161.7 MHz in vivo ^{31}P -spectrum of detached and perfused soybean root nodules. 20 K free induction decays were accumulated with a recycling time of 0.162 s. Detached nodules were split and perfused with a buffer solution containing 50 mM glucose, 0.1 mM CaSO_4 , 10 mM Mops (pH 7.5). O_2 was bubbled in the reservoir. X represents the resonance of the phosphate in the phosphocholine substituted β macrocyclic glucan.

with the procaryote as evidenced from the in vivo ^{31}P -spectrum of isolated bacteroids (Fig. 2A) and the perchloric acid extract of the free-living *B. japonicum*

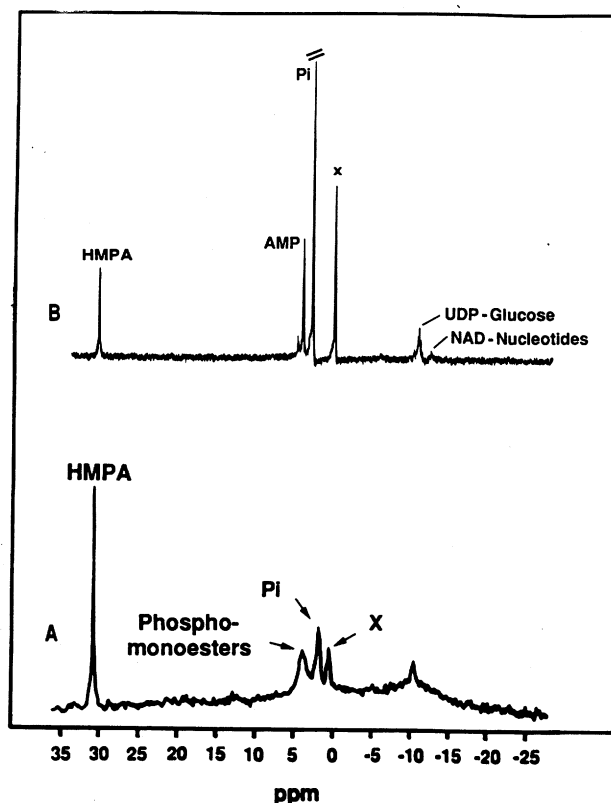


Fig. 2. (A) 161.7 MHz in vivo ^{31}P -NMR spectrum (20 K transients) of isolated bacteroids, perfused with the same buffer medium as Fig. 1. (B) proton decoupled ^{31}P -NMR spectrum of perchloric acid extract of *B. japonicum* USDA 110. The freeze-dried extracts were prepared as described by Rolin et al. [18]. HMPA, hexamethylphosphoramide (external reference at 30.73 ppm); X, P-resonance of the phosphocholine substituted β glucan.

USDA 110 (Fig. 2B). It was also present in the growth medium following the harvesting of this free-living *Bradyrhizobium*. The presence of this unidentified peak X in the ^{31}P -spectrum of the perchloric extract of different bacteria of the *Rhizobiaceae* family is summarized in Table I. Of those microorganisms examined, only *R. meliloti* 1020a and *B. japonicum* USDA 24, 110, and 138 appear to synthesize this unknown phosphodiester.

Two purification procedures were applied to isolate this compound from the free-living *B. japonicum* USDA 110. The first isolation was effected by elution of a freeze-dried perchloric acid extract from a Sephadex G-100 size exclusion column. In the second method, which gave superior separation, the freeze-dried sample was dissolved in water and eluted on a C18 silica reverse phase chromatography column. The column was washed with water and the unknown compound was then eluted off the column with a 30% methanol/water mixture. Freeze-drying of this material yielded a white powder. The average molecular weight of this material, determined by elution through a Sephadex G-100 size exclusion column, was in the range of 3.0–3.4 kDa and was verified by gel-filtration through a Bio-Gel P-4 column. Retention of standard compounds is somewhat variable on these columns due to differ-

TABLE I

Occurrence of the 0.44 ppm phosphodiester resonance in the ^{31}P -NMR spectrum of perchloric acid extracts of different bacteria of the *Rhizobiaceae* family

<i>R. leguminosarum</i> biovar <i>viceae</i> (2370)	not detected
<i>R. leguminosarum</i> biovar <i>trifolii</i> (2152)	not detected
<i>R. leguminosarum</i> biovar <i>phaseolii</i> (2676)	not detected
<i>R. meliloti</i> 1020a	detected
<i>B. sp. lotus</i>	not detected
<i>B. japonicum</i> USDA 110	detected
<i>B. japonicum</i> USDA 138	detected
<i>B. japonicum</i> USDA 24	detected

ences in shapes of the molecules, therefore the molecular weight determination using this methodology is only approximate (see Materials and Methods).

Further HPLC purification on an aminopropyl silica column resolved the mixture into PC and non-PC substituted glucans. Fig. 3A shows the HPLC analysis of the isolated glucan mixture extracted from *B. japonicum* USDA 110, and 3B the corresponding nodules obtained by infection of the Williams soybeans with *B. japonicum* USDA 110 after 6 w of growth. The retention volume peaks at approx. 18 min corresponding to the unsubstituted cyclic glucan represents 25% of the mixture in A and 50% of the mixture in B. The peak at approx. 27 min represents the PC substituted cyclic glucan as indicated in the Fig. 3. A sample of β -(1,3);(1,6) cyclic glucan obtained from K. Miller that has been reported to be a mixture of four ring sizes (C_{10} , C_{11} , C_{12} and C_{13}), co-chromatographed with the four early peaks centered around 18 min in Fig. 3A.

FAB mass spectra of the HPLC purified PC substituted glucan showed an $\text{M} + \text{H}$ molecular ion at 2272 corresponding to the mass of a 13 glucose containing cyclic glucan substituted with a single PC. A second ion approx. 3-times as large was observed with an $\text{M} + \text{H}$ of 2286. This latter value is 14 mass units higher than the expected mass and may be the result of intermolecular CH_3 migration (in the mass spectrometer) from one PC substituted molecule to another, as reported earlier [23], or a subtle structural feature not evident in the chemical or NMR data.

A carbohydrate compositional analysis by gas chromatography (following methylation) of the purified PC substituted material [21] indicated that glucose was the only carbohydrate present in this compound. A more detailed analysis of this oligosaccharide revealed the absence of detectable reducing sugar. The 125 MHz natural abundance ^{13}C -proton decoupled spectrum of this purified compound (Fig. 4) was similar to that of the extracellular β -(1,3);(1,6) glucan reported by Dudman and Jones [16]. The latter was found in the growth medium of *B. japonicum* strain 311b71a. The ^{13}C -NMR spectrum shown in the 2D heteronuclear COSY spec-

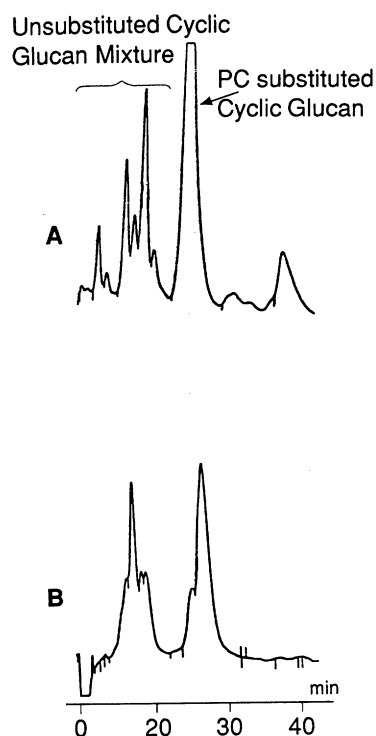


Fig. 3. HPLC (aminopropyl silica column) profiles of glucan mixtures derived from extraction of (A) *B. japonicum* USDA 110 and (B) soybean nodules containing bacteroids which originated as *B. japonicum* USDA 110. Conditions: 57:43 water:acetonitrile, flow 1 ml/min.

trum (Fig.4) exhibited resonances characteristic of a complex glucan. The resonances in the region of 62, 70, 74, 76, 85 and 104 ppm are assigned to C₆, C₄, C₂, C₅, C₃ and C₁, respectively, for the β -(1,3)-linked glucose residue (see Table III) [16,17]. A great deal of multiplicity associated with the C₃ (1-3) resonance is consistent with at least three different chemical shift environments for this residue at its linkage site. Likewise, the C₆ (1-6) resonance shows the same multiplicity. Resonances in the region of 69, 70, 74, 76 and 104 ppm are assigned to C₆, C₄, C₂, C₃ and C₁, respectively, for the β -(1,6)-linked glucan residues (see Table IV) [16,17]. Signals assigned to C₁ at 104.26 and 104.07 ppm indicate the oligosaccharide (1,3 and 1,6 linked) is a β -glucan. An additional resonance at 103.8 ppm corre-

sponds to the C₁ (1-3,6) at the branch point. A minor resonance observed at 82.00 ppm appears to be an impurity of some 1,2 glucan. The ¹³C-spectrum of our material also exhibited additional peaks at 55.19, 60.68 and 67.20 ppm which are characteristic of the N⁺ (CH₃)₃ choline, α -CH₂ and β -CH₂ resonances of phosphocholine, respectively. The ratio of ¹³C resonances obtained under NOE suppressed quantitative conditions showed the (1,3)- to (1,6)-linked ratio to be 1:1 \pm 12%, the (1,3,6) to (1,3) and (1,6) ratio to be 1:4.5 \pm 20% and 1:6 \pm 25%, respectively. Analysis of the phosphorus-containing cyclic glucan in boiling 0.5 M NaOH for 1 h 20 min lead to a \sim 70% conversion to three phosphomonoesters as determined by ³¹P-NMR. The three monoesters in the ratio of \sim 2:4:1

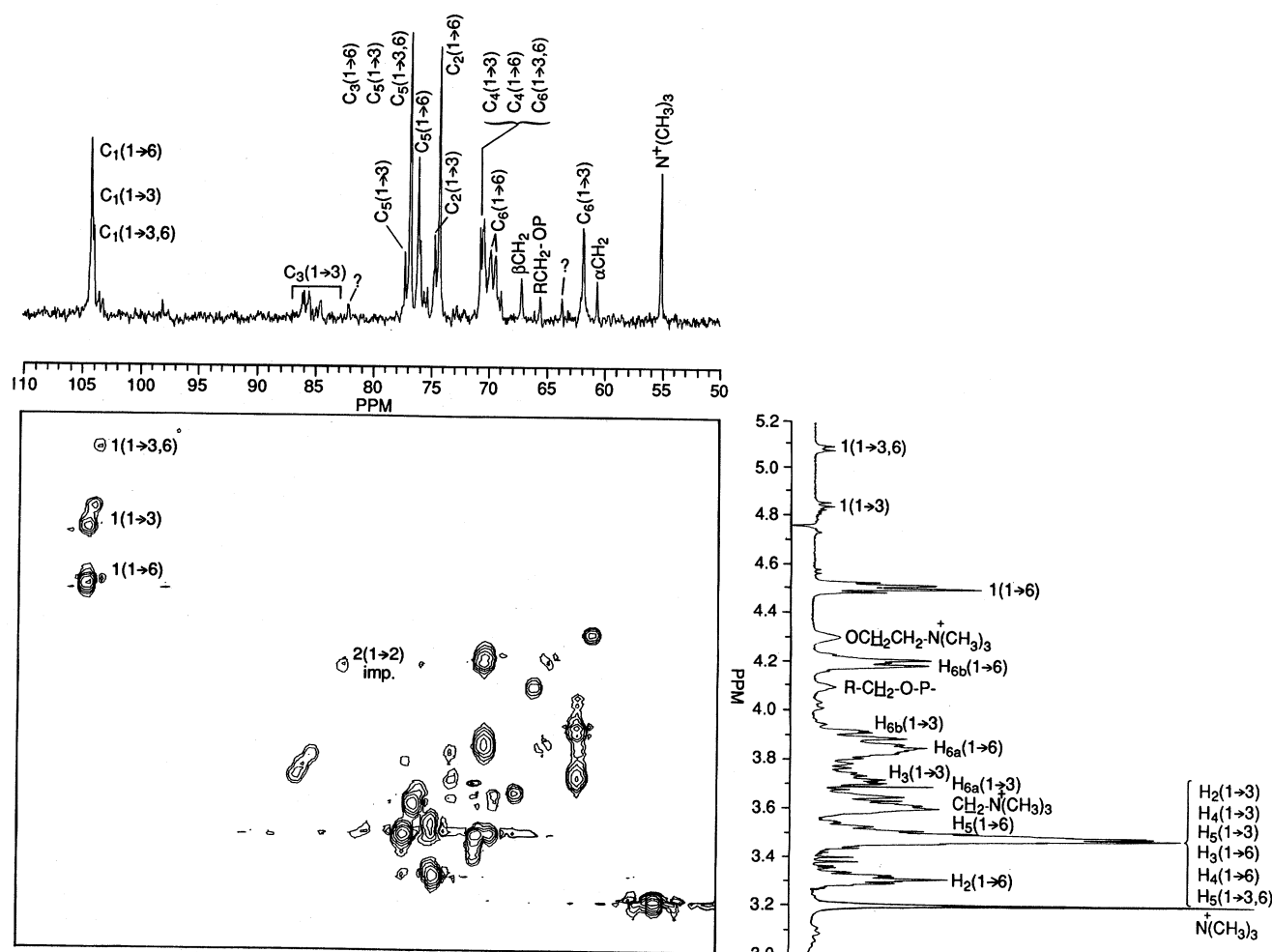


Fig. 4. Inverse ¹H-¹³C correlation 2D spectrum of the macrocyclic glucan obtained at 500/125 MHz with a 5 mm BB inverse probe and the BIRD X-nuclear decoupling sequence. The HDO resonance was suppressed by means of selective inversion of protons without directly attached carbons followed by a delay (0.90 s) which is chosen to obtain minimum FID intensity. Further suppression was achieved by means of the double quantum filter in the sequence which also eliminated ¹H signals from protons without directly attached ¹³C atoms. GARP1 ¹³C decoupling was used during the 0.47 s acquisition time. No irradiation of any kind was applied during the 0.5 s relaxation delay between transients. 512 transients were obtained for each 4 K slice of the data matrix, and 128 slices were obtained. The sweep widths in the ¹H and ¹³C domains were 4310 and 4032 Hz, respectively. The ¹³C (*T*₁) data columns were zero-filled to 256 data points prior to Fourier transformation to give a total data matrix of 4 K \times 256. Time proportional phase incrementation (TPPI) between successive data matrix slices was used to give pure absorptive (phase-sensitive) peaks and maximize resolution. Sine bell apodization, shifted by $\pi/3$, was used in both domains.

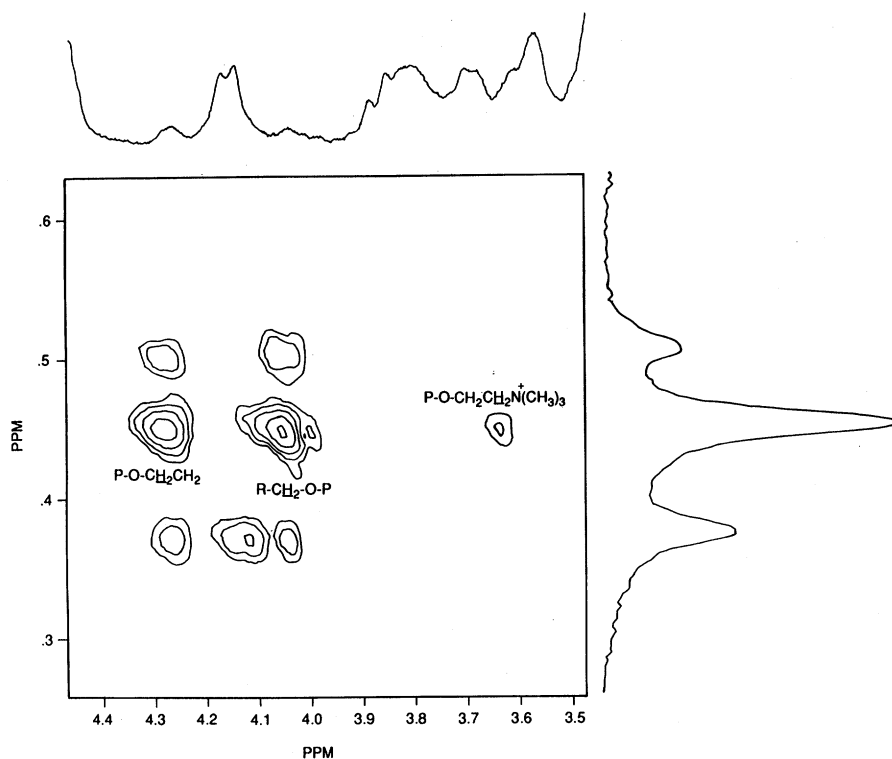


Fig. 5. 2D ^{31}P - ^1H COSY spectrum of the phosphocholine substituted β macrocyclic glucan obtained at 161.7/400 MHz with a 10 mm broadband probe and a 1.1 ml microcell insert. The spectral widths were each 1000 Hz in each dimension, 32 transients were used for each slice. total data matrix was 1 K \times 256. An average value of 6 Hz was chosen for $J_{\text{P-H}}$.

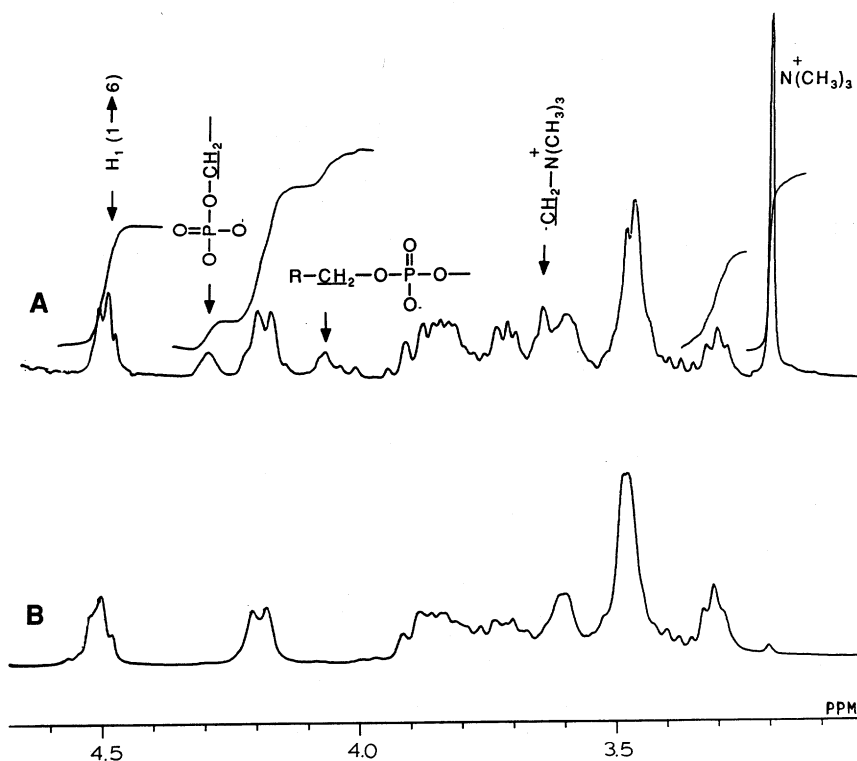


Fig. 6. 400 MHz ^1H spectra of HPLC (aminopropyl silica) purified phosphocholine substituted (A) and unsubstituted (B) cyclic glucan derived from *B. japonicum* USDA 110.

have ^{31}P resonances at 5.41, 5.35, and 5.21 ppm, respectively, at pH 6.8. Glucose 6-phosphate exhibits a ^{31}P chemical shift of 5.39 ppm under the same conditions. Analysis of the hydrolysis solution (after adjustment of the pH to 8.0) with choline oxidase showed the presence of choline. It was difficult to obtain a quantitative estimation of the choline concentration due to the high concentration of NaCl remaining in the neutralized solution following the hydrolysis of the choline group however, the analysis corresponded to approximately one choline group per each 2500 molecular weight unit.

The 2D heteronuclear ^{31}P - ^1H COSY spectrum (Fig. 5) of the HPLC isolated PC substituted compound showed three ^{31}P resonances in the ratio of approx. 1:2:4 indicating that phosphorus can occupy three different chemical environments or points of attachment in this structure. These shifts show a small pH dependence (0.15 ppm over a pH range from 5.5 to 10.4). The data in Table II shows that each ^{31}P -resonance is coupled to a glucose H6 at 4.08 ppm [24] which in turn is coupled to a C_6 -resonance at 65.5 ppm (Fig. 4). The ^{31}P is also coupled to resonances at 4.30

TABLE II

^{31}P and corresponding ^1H and ^{13}C chemical shift * assignments for the phosphocholine substituted glucose residues

C		H		P
C_6	65.50	$\text{H}_{6a,b}$	4.08	
αCH_2	60.68	αCH_2	4.30	0.39, 0.44, 0.54
βCH_2	67.20	βCH_2	3.62	

* ppm.

and 3.62 ppm corresponding to $\alpha\text{-CH}_2$ ($^3J_{\text{PH}}$) and $\beta\text{-CH}_2$ ($^4J_{\text{PH}}$) resonances of a choline moiety (Fig. 4). The coupling to $\beta\text{-CH}_2$ is only seen for the major product in this 2D spectrum because of the lower levels of the other phosphorus substituted derivatives, however, the coupling ($^4J_{\text{PH}}$) of the two minor phosphorus peaks was also observable at lower spectral thresholds (data not shown). The αCH_2 and βCH_2 protons in turn yield cross peaks with the appropriate choline ^{13}C -resonances seen at 60.68 and 67.20 ppm, respectively, in the 2D ^{13}C - ^1H spectrum (Fig. 4). The choline methyl resonance at 55.19 ppm shows a cross

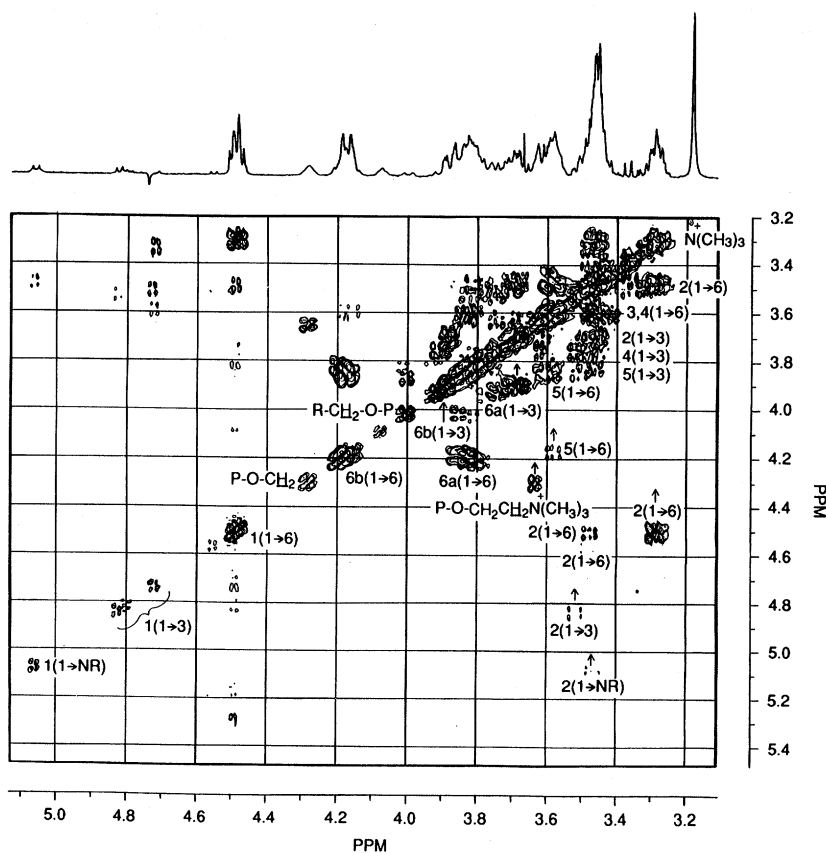


Fig. 7. 2D double quantum filtered phase-sensitive ^1H homonuclear COSY spectrum of the phosphocholine substituted β macrocyclic glucan obtained at 500 MHz using a spectral width of 3787 Hz and presaturation of the HDO resonance. 256 slices were obtained in the T1 domain, with zero filling to 1 K prior to Fourier transformation. The overall data matrix was 4 K \times 1 K. Time proportional phase incrementation (TPPI) was used between successive slices to yield the phase sensitive 2D spectrum. 32 transients with 4 dummy scans were acquired for each slice. The HDO resonance was presaturated for 1.1 s prior to each acquisition. The $\pi/2$ and π pulse widths were 7, 6 and 15.1 μs , respectively.

TABLE III

^{13}C and corresponding ^1H chemical shift assignments for the (1,3)-linked glucose units

	ppm		ppm
C ₁	104.0, 103.8	H ₁	4.70, 4.82
C ₂	74.2	H ₂	3.44
C ₃	84.6, 85.6, 86.0, 86.2	H ₃	3.73
C ₄	70.8	H ₄	3.49
C ₅	76.81	H ₅	3.49
C ₆	61.94	H _{6a,6b}	3.88, 3.72

peak with the CH₃ proton resonance at 3.20 ppm (Fig. 4). Fig. 6 shows the upfield portion of the 1D ^1H spectra of the (A) PC and non-PC substituted cyclic glucans purified by HPLC, respectively. Resonances observed at 4.05, 3.62 and 3.20 ppm correspond to the choline substituent and the resonance at 4.17 ppm the phosphorylated H6 in (A) as reported previously by Hauser et al. [25]. These shifts are clearly absent in the unsubstituted glucan mixture (B). Tables III and IV list all the ^1H chemical shift assignments for the PC substituted cyclic glucan, based on the 2D double quantum filtered phase-sensitive ^1H homonuclear COSY spectrum shown in Fig. 7. All anomeric linkages were found to have the β configuration ($J_{1,2} = 7.8$ Hz) [26].

As described above, the ^{13}C -NMR spectrum of the purified PC substituted glucan of *B. japonicum* USDA 110 contains features indicative of the presence of β -(1,3) and β -(1,6) glycosidic linkages. The presence of (1,6), (1,3,6) and (1,3) glycosidic linkages within the structure was confirmed by GLC-MS analysis of the permethylated alditol acetates derived from the oligosaccharide [21]. Integration of the NOE suppressed 1D ^{13}C -spectrum and methylation analysis indicated that the ratio of β -(1,6):(1,3):(1,3,6): non-reducing terminal glucose residues was 6:5:1:1, respectively.

Based on the ratio of the ^{13}C CH₃ choline resonance (normalized for 3 carbons) we obtained an anomeric carbon/choline ratio of 1:13. No unsubstituted reducing end anomeric carbons were observed by NMR. Indeed, resonances at 92 to 96 ppm corresponding to the C₁ resonance of a reducing glucose residue were absent in the ^{13}C -NMR spectrum as were any

TABLE IV

^{13}C and corresponding ^1H chemical shift assignments for the (1,6)-linked glucose units

	ppm		ppm
C ₁	104.2	H ₁	4.49, 5.07 (terminal gl)
C ₂	74.2	H ₂	3.31, 3.44, 3.47 (terminal gl)
C ₃	76.8	H ₃	3.48
C ₄	70.8	H ₄	3.48
C ₅	76.8	H ₅	3.60
C ₆	69.0, 69.5, 69.9	H _{6a,6b}	3.82, 4.19

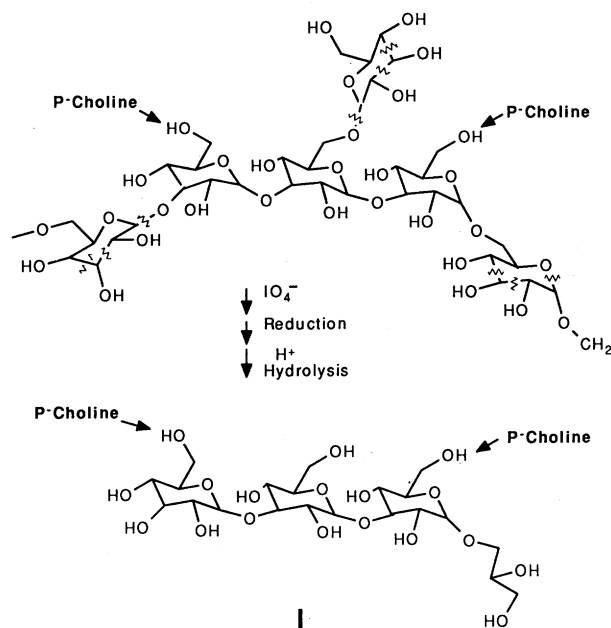


Fig. 8. Smith degradation of phosphocholine substituted cyclic glucan.

carbonyl resonances in the 180 ppm region. Also, no reducing sugar residues were detected by end group analysis in the oligosaccharide preparation derived from *B. japonicum* USDA 110.

Following the Smith degradation of the glucan (Fig. 8) we isolated a C₁ glycerol substituted trisaccharide whose GLC analysis and ^{13}C -spectrum corresponded to the that of the glycerol substituted laminaritrise (structure 1, Fig. 8) [27]. The fact that only this oligosaccharide was isolated clearly indicates that the periodate resistant residues (the β -(1,3)-linked glucoses) are in units of 3 and are separated by the (1,6) glucose units that were fully oxidized. Resonances were also observed for C₁, C₂ and C₃ glycerol at 62.7, 72.0 and 71.0 ppm, respectively. Shifts associated with the phosphorylated C₆ of glucose and choline groups were observed at 65.0 ppm (C₆-PO₄⁻), 59.0 ppm (αCH_2), 65.2 ppm (βCH_2) and 55.1 ppm ($(\text{CH}_3)_3\text{N}^+$) [25]. The ratio of each (CH₃)₃N⁺ group resonance to anomeric carbon resonance was 0.13 indicating that approx. 80% of the single choline phosphate substitution occurs on one of the five C₆-OH groups of the ring β -(1,3)-linked glucose residues. The remaining 20% substitution must reside on the C₆-OH of the point non-reducing terminal glucose unit. However, since this glucose unit is removed in the Smith degradation the phosphocholine group is not observed (see Fig. 8).

Discussion

In the present study, we have demonstrated that *B. japonicum* USDA 110 synthesizes a a single branched

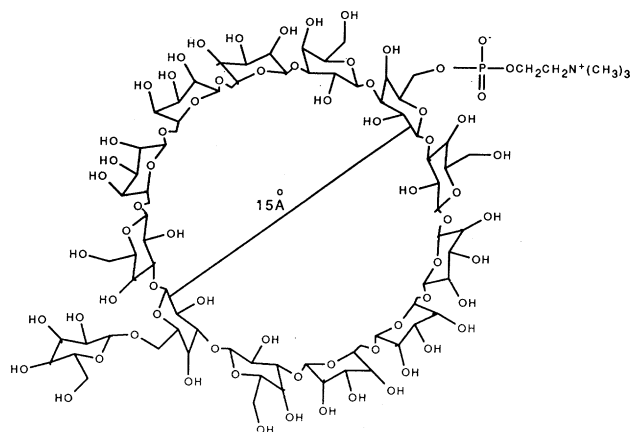


Fig. 9. Schematic representation of the structure of the β -(1,3);(1,6) phosphocholine substituted macrocyclic glucan synthesized by *B. japonicum* USDA 110.

phosphocholine substituted β -(1,3);(1,6) 12 membered macrocyclic glucan. The most probable structure based on the results of 2D-NMR, methylation analyses, end group analysis, mass spectrometry, HPLC and the Smith degradation is shown in Fig. 9. We have depicted the structure of this compound for simplicity, as containing 13 glucose residues with two repeating units each having 3β -(1,3) and 3β -(1,6) glucose residues. Alternatively, the structure might also contain segments in which the β -(1,6) units are found in groups of 2 and 4 or 5 and 1 although this is somewhat less likely to occur. Based on the Smith degradation results it is unequivocal that the three β -(1,3)-linked glucose residues are contiguous and separate the β -(1,6) residues. One sequence of β -(1,3)-linked glucose units contains a glycosidically linked glucose at the C₆OH position. The PC appears to be preferentially substituted on the C₆OH of the remaining one of (1,3)-linked glucose units (see Fig. 9). The multiplicity (minimum of 3 resonances) of the ^{31}P -resonance of the PC cyclic glucan (Fig. 5) indicates that the PC substitution sites do not have chemically equivalent environments.

It is possible that the phosphocholine substituted β glucan of *B. japonicum* USDA 110 described in the present study is structurally related to the extracellular glucans of *B. japonicum* 3I1b71a that has been previously described by Dudman and Jones [16]. Likewise it is possible that the cell-associated unsubstituted glucan of *B. japonicum* USDA 110 and *Bradyrhizobium* sp. strain 32H1 described by Miller et al. [17] are also similar in structure. All these unsubstituted cell-associated or extracellular glucans are composed of β -(1,3) and β -(1,6) glycosidic linkages. However, unlike the presently reported structure, the extracellular polysaccharide contained twice as many (1,3)-linked as (1,6)-linked glucose units [16]. In addition, the extracellular glucans of the strain 3I1b71a appear to be larger

(mol. wt. of approx. 4500 by gel-filtration chromatography), whereas the cell-associated cyclic glucan of strain USDA 110 and 32H1 reported by Miller et al. [17] (11 to 13 glucose residues) appears to be similar in size to the glucan described in this paper (13 glucose residues). It is conceivable that the previously reported glucans [16,17] were also similarly substituted. However, the methods by which they were isolated probably resulted in the removal of the phosphocholine substituents. More specifically, the earlier isolations were performed by either evaporation of the extracellular broth [16] or extraction of the microorganism with chloroform/methanol [17]. Our method of isolation differs from the former in that we ground the frozen bacteria with perchloric acid solution at liquid nitrogen temperature to eliminate any enzyme activity (i.e., phosphodiesterase or phosphatase) prior to further purification. Thus, we have observed both the PC and non-PC substituted cyclic glucans. In essence, our purification was monitored by ^{31}P -NMR and in so doing we altered the conditions to be certain we would isolate the PC containing polymer. It is interesting to note that although the unsubstituted cyclic glucan appears to exist in at least four ring sizes (10–13) [17], the PC substituted counterpart is restricted to the 12 membered ring with a single branch. From the recent work reported by Geiger et al. [28] on β -1,2 cyclic glucans derived from *R. meliloti* 1021 it appears that unsubstituted cyclic glucans are precursors to the phosphorylated derivatives. However, the functional and metabolic connection between these glucans is yet to be discovered.

The cyclic glucan reported in this paper is the first PC substituted β -(1,3);(1,6)-linked glucan to be observed in *Rhizobia*. β -(1,3) Glucans are extremely widespread in fungi, algae and plants, but little is known of their distribution in bacteria. The extracellular polysaccharides of the *Rhizobiaceae* family are a subject of considerable interest and speculation because of the role they may play in the specificity of symbiosis between *Rhizobium* species and their plant hosts [16]. The possibility that the presently described cyclic glucan could be involved in this biological process is especially intriguing because other cyclic glucans of similar structures (cyclic β -(1,2) periplasmic glucans) have been found to possess considerable biological activity [7,12–14]. It is conceivable that the 'hydrophobic' character of the phosphocholine substituted β -(1,3);(1,6) cyclic glucan, observed during its purification may reflect an important property of this macromolecule with regard to its association in a membrane-environment. Considering that this cyclic polysaccharide contains a phosphocholine group argues that it is membrane derived as are the phosphoglycerol substituted 1,2 cyclic glucans found in the periplasm of *Escherichia coli* [29] *Agrobacterium tumefaciens* [30] and *Bradyrhizobium* sp. strain 32H1 [17].

faciens [7] and *R. meliloti* 1021 [28]. Tully et al. [11] have provided evidence for the possible role of a β -glucan in osmotic regulation of *B. japonicum* USDA 110. We have examined one fraction of these isolated glucans supplied by Tully and found it to have the same spectrum as the phosphocholine substituted material reported here.

Detailed molecular modelling with energy minimization molecular model calculation of the choline-phosphate substituted β -(1,3);(1,6) macrocyclic glucan. 2D-NMR water exchange experiments and antibody preparations are in progress to help establish the location as well as the function of this unprecedented structure in symbiotic nitrogen fixing bacteria.

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